EFFECT OF SEMI-CONTINUOUS OPERATION MODE PARAMETERS ON BACTERIAL CELLULOSE AND BIOMASS PRODUCTION

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Bacterial cellulose (BC) has advanced properties, such as high crystallinity, high molecular weight and high purity. Therefore, it can be used extensively in the cosmetic and pharmaceutical industries. Because of the importance of BC, an economical production process is needed. In this study, BC production in a static culture was investigated with varied semi-continuous process parameters, including the volume changing ratio (%; ml/ml), the initial glucose concentration (%; g/100 ml) and the surface area/volume ratio (cm^{-1}). BC and cell dry weight (CDW) were measured, and BC was characterized by tensile strength, SEM and FT-IR. BC productivity reached 0.36 g/L/day after 6 days of incubation. Semi-continuous process parameters were investigated by changing the medium every 6 days. The optimal parameter values for BC production were a volume changing ratio of 60%, an initial glucose concentration of 30% and a surface area/volume ratio of 0.85 cm^{-1}. BC production reached 15.6 g/L in this semi-continuous process, which was 7.5-fold greater than in the batch process. This semi-continuous process is a good candidate for industrial-scale BC production.

Keywords: bacterial cellulose (BC), semi-continuous process, volume changing ratio, incubation time period, glucose concentration, surface area/volume ratio

INTRODUCTION

Cellulose, the most abundant polymer in nature, is uniformly formed from glucose monomers linked by β(1–4) glycosidic linkages.[1] Unlike plant cellulose, bacterial cellulose (BC) lacks impurities, such as hemicellulose and lignin, which eliminates the necessity of harsh chemical treatments to remove them.[2] Although bacterial and plant celluloses share similar chemical structures, BC has certain advantageous properties, such as its purity, high crystallinity, high tensile strength, high biodegradability and water-holding capacity.[3,4] Due to these characteristics, BC has various industrial applications in the textile, pharmaceutical, biotechnology, food and paper industries.[5-10] BC has been produced using different species of bacteria, such as *Gluconacetobacter* (*Acetobacter*), *Agrobacterium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, *Salmonella*, and *Escherichia*; among these species, *Gluconacetobacter xylinus* is the most widely used and efficient producer.[11-13]

To enhance the variety of its uses and increase the efficiency of its production, researchers have focused on manipulating the synthesis of BC with chemicals, different operating systems, and new bioreactor designs.

Currently, static[4,14] and agitated culture[15,16] methods are used for the production of BC. A static culture is a commonly used method, and pellicle structures can be acquired starting from the surface. The shape of the pellicle structure renders static culture-produced BC more commercially feasible because agitated culture-produced BC is a slurry, which limits its applications.[17] Because the formation of BC is important for wound dressing and skin tissue engineering, structural deformation can limit its application. To use BC for wound dressing, the BC product must be smooth and film-like to apply

to wounds.[18]

To achieve commercial-scale production, different bioreactor types have been suggested. Kralisch *et al*.[15] developed the Horizontal Lift Reactor (HoLiR) for planar bacteria-produced nanocelluloses. In addition to the homogeneity problem, for removing the BC product from agitated bioreactors, BC also possesses low mechanical strength, reducing its application efficiency.[17] Hornung, Ludwig and Schmauder[19] developed a bioreactor with an aerosolized glucose spray to evenly distribute the medium and increase bacterial development. Many researchers have successfully developed bioreactors, but their operating systems also need improvement.

Generally, fed-batch or batch cultures have been used as operating conditions for BC production.[12,19-22] Fed-batch and semi-continuous strategies are used to overcome the longer cultivation times and lower production levels achieved in static cultures, as well as facilitating the industrial applications of BC. Different types of carbon sources have been assayed to increase production. To produce BC efficiently, the choice of BC production medium and composition are also important.[23,24]

Shezad, Khan, Khan and Park[20] studied a fed-bath system that used waste from fermentation broth (WTFB) as the nutrient supply, and compared with batch cultivation, a 2- to 3-fold increase was observed after 30 days of production.[4] Krystynowicz *et al*.[25] observed fermentation conditions using the Hestrin and Schramm (HS) and Yamanaka medium and obtained 0.015 g/l/h productivity. Lin and Cheng[17] investigated BC production using a corn steep liquor and fructose (CSL-Fru) medium with a plastic composite support (PCS) in a semi-continuous system to increase cell adhesion; in a 5-day cultivation, 0.24 g/L/day productivity levels were achieved.[26] Ruka, Simon and Dean[27] studied various media with different feed rates and achieved 10 g/L productivity in a 7-day cultivation, although this method was not cost-effective, and the optimal medium was undefined. Sugarcane molasses have also been used as a carbon source for BC production.[28-30]

Recently, semi-continuous operation modes have been studied to improve yield. Lin *et al*.[26] investigated a semi-continuous process with *G. xylinus* and achieved BC productivities as high as 0.24 g/L/day in a 5-day cultivation.[26] Çakar, Özer, Aytekin and Şahin[11] produced BC in a semi-continuous operation mode with a static culture using a molasse medium, and the highest BC production obtained was with a VCR of 1/2 and a 7-day incubation. However, the effects of the other parameters still need to be clarified.

Traditionally, BC production is performed using static conditions,[31] but there are many parameters that need to be optimized, such as the amount of glucose in the medium, the surface area/volume ratio, the inoculation ratio, the incubation day interval, and the VCR. Because these parameters affect productivity, BC production in a static culture in a semi-continuous mode has been suggested.[26]

In this study, BC was produced using a static culture of *Gluconacetobacter xylinus* FC01 in semi-continuous operation with M1A05P5 medium. The VCR, glucose concentration in the medium, incubation day interval, and surface area/volume ratio were varied to determine their effects on the production yield. BC samples were characterized using SEM, FT-IR and DSC methods. Cell dry weight (CDW; g/L) and BC (g/L) were also quantified.

EXPERIMENTAL

Bacterial strain and culture

G. xylinus FC01 was cultured on Hestrin-Schramm agar plates for 2 days before isolation for this study.[32] Isolated *G. xylinus* was cultured in a medium containing 10, 30, or 50% glucose; 7 g/L peptone; 10 g/L yeast extract; 1.575 g/L acetic acid; 5 g/L ethanol; the pH was adjusted to 5 with HCl or NaOH. The cultures were grown in their various media in differently sized bottles to maintain a 0.2, 0.85, or 1.5 cm^{-1} surface area/volume ratio. The VCR was adjusted to 33%, 50%, and 66% (v/v) over 24 days.

Bacterial cellulose analysis

The samples collected after 6 days were centrifuged at 3,600 rpm and 4 °C for 20 min to remove the supernatant. The BC samples were stored at -80 °C overnight and subsequently freeze-dried. The samples were treated with 0.3 M NaOH at 80 °C for 1 h to degrade any remaining bacteria. The cell dry weight (CDW; g/L) was calculated from the weight difference after the second freeze-dry.

Fourier transform infrared spectroscopy

A Perkin-Elmer Spectrum 100 Spectrometer was used for FT-IR spectroscopy. Scans were performed in the 4,000-450 cm^{-1} range. The Spectrum software was used to normalize the baselines of each spectrum.

Scanning electron microscopy

The samples were coated with gold particles for SEM observation and imaged using a Carl Zeiss EVO-

40 instrument under vacuum at a high potential, 10 kV.

Mechanical properties of BC

The tensile strength and Young's modulus of the BC were analyzed using an Instron 5900. Two different methods, based on the dry and wet states, were performed to analyze the BC films. Then, the BC was freeze-dried, and the samples (0.2 g) were weighed and pressed at 1,000 psi to prepare a flat film. Then, the BC films were cut into 4 x 1 cm samples. The BC films were placed into the device and tested at 0.1 N/min and at room temperature. Wet BC was analyzed with the same method, minus the drying step. The Young's modulus was calculated using the following equation:

$$\text{Young's modulus} = \frac{\text{Stress}}{\text{Strain}} = \frac{F/(w \times t)}{\Delta L/L_O} \quad (1)$$

In this equation, F is the force (N), w is the width of the film (mm), t is the thickness of the film (mm), ΔL is the extension length of the film under force (mm), and L_O is the initial length of the film (mm).

RESULTS AND DISCUSSION

Optimal incubation interval

G. xylinus was incubated in a medium containing 10 g/L glucose in batch operation mode and sampled daily over 24 days to analyze the concentration and productivity of BC and the CDW (Fig. 1). The highest BC concentration, 2.05 g/L, was observed on the 12th day. Although the concentration was observed at its highest point on the 12th day, the productivity reached its maximum value on the 6th day. The chosen incubation day interval was 6 days to facilitate the investigation of other parameters. Productivity is important at the industrial scale; therefore, productivity was prioritized, and a 6-day incubation interval was selected with semi-continuous production. Hornung, Ludwig, Gerrard and Schmauder[33] chose a 10-day interval because it has been reported that after that day, mass transfer limitations start to affect cellulose production in HS medium. Çakar, Özer, Aytekin and Şahin[11] selected a 7-day interval with a molasse medium in the batch mode. Using a 5-day interval, Lin *et al.*[26] achieved a productivity of 0.25 g/L/day with a rotating-wall bioreactor and CSL-Fru medium. The selection of an appropriate incubation day interval in the literature varies due to the choice of reactor and the condition of the microorganism. Because the parameters of the above studies differ from our study, BC production reached maximum levels on different days. In this study, cellulose productivity was the prime consideration for the selection of the incubation interval. The effect of the different VCRs was negligible at the beginning of the incubation, but increased toward the end of the 24-day incubation, and the total BC production achieved with a 30% VCR was lower than that with 50 and 60% VCRs. These results indicate that VCRs of 50 and 66% yield significantly higher BC production outcomes and should be considered for semi-continuous processes. However, the selection of the VCR should incorporate economic considerations. Therefore, a 50% VCR can be used because the volume of the spent medium is less than with a VCR of 66%, which reduces medium costs.

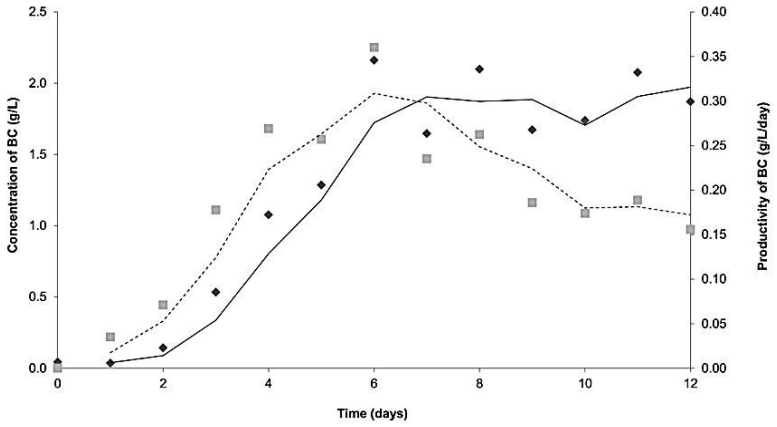

Figure 1: Evolution of BC concentration (- ; g/L) and productivity (---; g/L/day) over time in a batch process

Table 1
Production of BC (g/L) with varied parameters in a semi-continuous process

Sets	Volume changing ratio (%)	Glucose concentration (%)	Surface area/ volume ratio (cm^{-1})	Incubation days			
				6	12	18	24
1	33	30	0.85	2.07	3.12	4.36	6.23
2	66	30	0.85	4.03	6.99	11.23	15.54
3	50	30	0.85	3.00	7.60	11.77	15.63
4	50	30	0.2	0.91	1.72	2.14	3.29
5	50	30	1.5	3.03	4.48	5.76	9.58
6	50	10	0.85	1.46	2.85	3.88	6.01
7	50	50	0.85	3.23	6.18	8.61	12.05

Effect of surface area/volume ratio on BC

Because oxygen transfer is important for the efficiency of BC production, the SVR must be considered.[21] This parameter directly affects the thickness of the BC layer. The effect of the SVR was measured at three different values, 0.2, 0.85, and 1.5 cm^{-1}, with differently sized bottles. The lowest BC production (3.29 g/L) was obtained with an SVR of 0.2 cm^{-1}, the highest BC production (15.63 g/L) was obtained with 0.85 cm^{-1}, and BC production with 1.5 cm^{-1} achieved an intermediate value (9.58 g/L) after 24 days (Sets 3, 4 and 5 in Table 1). Although BC production with an SVR of 1.5 cm^{-1} was higher than that with 0.2 cm^{-1}, it was difficult to obtain a BC film with the former. If the SVR is very high, it is difficult for bacteria to produce cellulose in layers, and the BC structure can lose its rigidity. To increase BC production efficiency and yield a film suitable for downstream applications, an SVR of 0.85 cm^{-1} is ideal.

Okiyama, Shirae, Kano and Yamanaka[13] showed that production directly depends on the air/liquid surface area ratio if the depth is less than 4.5 cm. Due to the depth and surface area, the effect of the surface area/volume ratio was observed carefully. The optimal SVR found by Krystynowicz *et al*.[25] was 0.71 cm^{-1} using HS medium, whereas Ruka, Simon and Dean[27] suggested an SVR of 0.57 cm^{-1} with HS-glucose medium. Our results are more similar to those of Krystynowicz *et al*.[25] than to those of Ruka, Simon and Dean.[27] This difference, with respect to the 0.57 cm^{-1} value, may originate from differences in the medium composition not mentioned in that study. The increase in production we observed with an SVR of 0.85 cm^{-1} may be due to the ethanol and acetic acid in the medium. These components may prevent the growth of acetic acid bacteria and significantly affect BC production.

Effect of glucose concentration on BC

Because the substances in the medium directly affect the production of BC, different glucose concentrations were assayed with a 6-day interval over 24 days. Glucose media with 10, 30 or 50 g/L were prepared, and the resulting BC production levels were observed (Sets 3, 6 and 7 in Table 1). Although it was assumed that production would increase with the addition of more glucose, the 50-g/L medium resulted in less BC production than the 30-g/L medium. The lowest BC production was achieved with 10 g/L glucose. Glucose concentrations higher than 30 g/L exerted a negative effect on BC production, likely due to glucose inhibition.

Biomass generation

The relationships between all of these parameters contribute to the production outcome, although it is difficult to separate the individual effects of these conditions. Figure 2 presents these operating condition parameters for comparison. Within Sets 1, 2 and 3, the 50% VCR rate (Set 3) appears optimal because it yielded the highest levels of cell dry weight. A VCR of 33% (Set 1) yielded a lower CDW than a VCR of 50% (Set 3); however, production with a VCR of 66% (Set 2) yielded the lowest production level. Set 2 indicates that the highest VCR required the removal of too many microbes from the culture and negatively influenced production. Sets 3, 6 and 7 revealed the effects of glucose concentration on production. A glucose concentration of 30 g/L (Set 3) was better for production, according to the observed CDW. Lower levels of glucose decreased production (Set 6), but not as much as increased levels of glucose (Set 7). For *G. xylinus*, 50 g/L glucose is a stressful environment (Set 7), and growth inhibition was clearly observable.

Lowering the SVR values also lowered

production, according to the CDW values of Sets 3, 4 and 5. The high CDW obtained with a 50% VCR, 30 g/L glucose and an SVR of 1.50 cm^{-1} SVR indicates that these conditions are optimal for BC production. As the SVR was increased, the CDW rose gradually, and the highest CDW was observed with an SVR of 1.50 cm^{-1}. Because BC is generated slowly, when BC layers are formed, O_2 transfer from the surface to the culture starts to be limiting, and BC layers cannot grow thicker. Because small SVR values enable more rapid thickening than the larger SVR values, the growth of the microorganisms is slower with smaller SVRs.

Due to the complexity of multiple parameters and their interactions, further investigations should be performed to expand on this study.

Characterization of BC

The tensile strength of BC was characterized by its Young's modulus in the dry and wet state. BC is widely used in the cosmetic and pharmaceutical industries, and for these applications, BC is generally in contact with water or solvents. To observe the drying effect on BC after cell removal, BC samples were prepared in two different states. The preparation of samples in the first state, dry BC, is based on a standard method applied after BC purification. The BC was freeze-dried and stored at 4 °C until further use. The preparation of samples in the second state, wet BC, used the same method as for dry samples but without the drying process. The BC was stored in water at 4 °C until further use. As shown in Table 2, Young's modulus of wet BC was higher than that of dry BC.

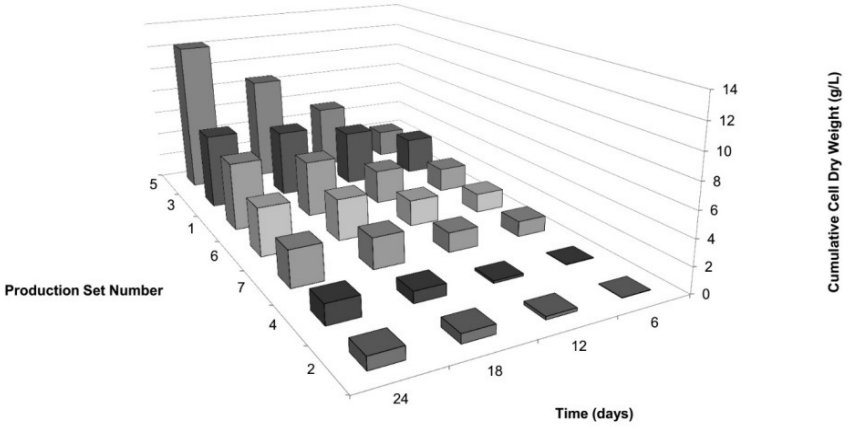

Figure 2: Cell dry weight concentration in a semi-continuous process using different sets of operating parameters (the properties of the sets are shown in Table 1)

Table 2
Tensile strength of BC before and after drying

Set	BC (g/L) on 24th day	CDW (g/L) on 24th day	Young's modulus (MPa)	
			Wet membrane	Dry membrane
1	6.23	5.40	249	175
2	15.54	0.95	357	298
3	15.63	6.02	301	267
4	3.29	1.55	209	134
5	9.58	12.30	168	101
6	6.01	3.82	221	123
7	12.05	2.85	287	242

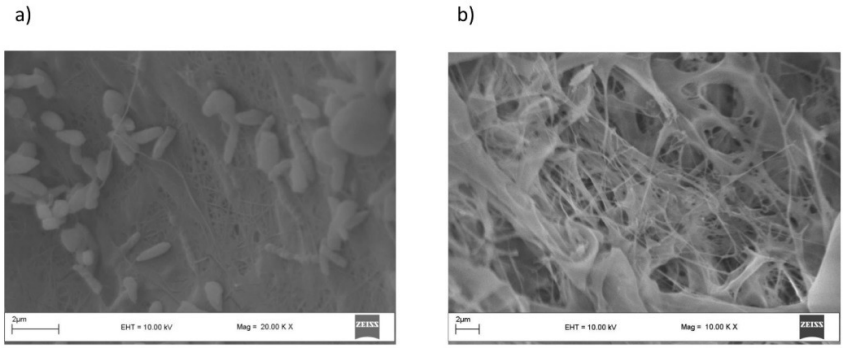

Figure 3: SEM images of BC before (a) and after (b) drying

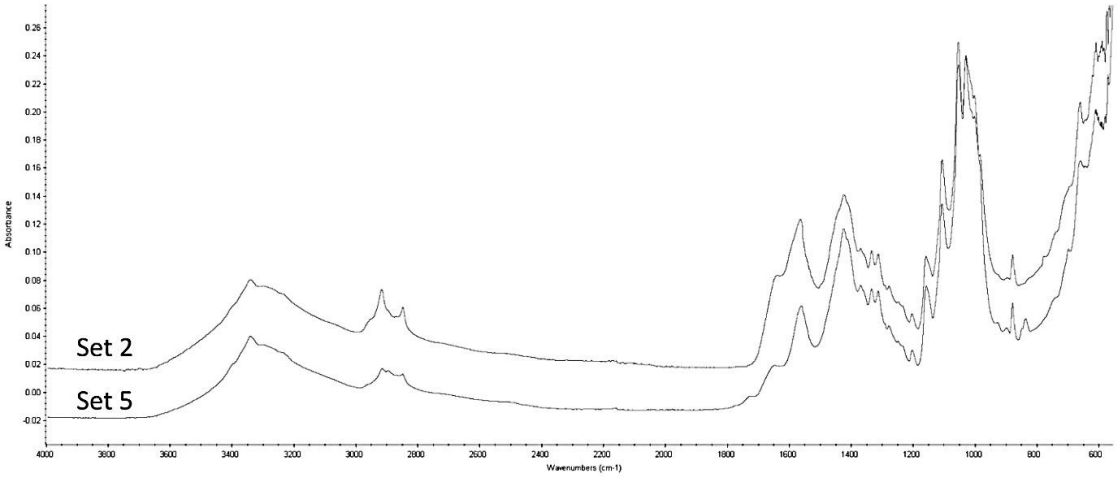

Figure 4: FT-IR spectra of BCs from Sets 2 and 5

A strong interaction between the amount of BC and Young's modulus was observed. The CDW has an important effect on Young's modulus of BC. The BC amounts achieved with Sets 2 and 3 are comparable; however, Set 3 yielded a higher CDW, and Young's modulus of Set 3 BC was lower than that of Set 2 BC. The lowest Young's modulus was observed in BC produced *via* Set 5, which yielded the highest CDW. During the generation of BC, microorganisms and their debris become entrapped in BC layers. Increasing the amount of CDW creates gaps in the BC layers that result in a non-uniform composition of the BC fibrils. Therefore, Young's modulus was lower with a high CDW than with a low CDW. The drying process also affected fibril strength, as freeze-drying has a crucial effect on the material; the long and smooth fibril structure was damaged during drying (Fig. 3). Because of this effect, dry BC exhibited lower Young's modulus than wet BC.

The morphological structures of the BCs are shown in Figure 3. The SEM images are significantly different before and after cell removal. A microorganism (or its debris) is clearly observable in Figure 3a, and the BC surface is smooth and dense. However, after an alkali treatment was performed to remove cells and other medium components, large pores and BC fibrils were obtained (Fig. 3b).

FT-IR is a frequently used method for the characterization of cellulose and other polymers. The FT-IR spectra of the BC samples are shown in Figure 4. Cellulose and the BCs produced in Sets 2 and 5 were chosen for FT-IR analysis. The BCs from Sets 2 and 5 exhibited the highest and lowest Young's modulus values, respectively. Based on the cellulose spectrum, the BCs of Sets 2 and 5 exhibit similar patterns in carboxylic acid and carboxylate groups, hydroxyl groups, ether bonds and hydrogen bonds. These results indicate that the BCs from both processes were cellulose.

CONCLUSION

BC production in a semi-continuous process was increased 7.5-fold *versus* a batch process. The optimal parameters for BC production included a 30% glucose concentration and an SVR of 0.85 cm^{-1}. However, the optimal results for VCR differ, depending on the desired process outcomes. A VCR of 50% yields a higher BC production than a VCR of 66%, but the resulting CDW with the latter was low, which eases purification and reduces the use and cost of harsh chemicals. However, substrate consumption was higher with a VCR of 66%. Therefore, total process costs should be considered when determining the optimal operating conditions. Moreover, if the downstream application does not require dry conditions, the BC should be prepared and stored in the wet state to maintain high quality.

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